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FOREWORD

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Grant DMAD17-96-1-6221, Role of USF in breast cancer

Michele Sawadogo, Ph.D., Principal Investigator

Progress report: September 1, 1996-August 31, 1998.

INTRODUCTION

The purpose of this grant was to investigate the role of the USF proteins in breast cancer. The major reason to initiate these studies was the demonstrated antagonism between the cellular function of USF and that of the Myc oncoproteins. Myc overexpression, whether due to gene translocation, amplification or increased message stability, is known to play a key role in cancer progression (1, 2). For breast tumors, Myc overexpression is a very strong indicator of an increased risk of relapse, especially in the case of node-negative patients (3, 4).

Myc and USF both belong to the basic-helix-loop-helix-leucine zipper family of transcriptional regulators. They also share a common DNA-binding specificity for sites containing a 5' CACGTG 3' core sequence (5-8) and are both indispensable during embryonic development (9, 10). However, the Myc proteins promote cellular proliferation and their overexpression leads to uncontrolled growth (1). In contrast, studies in our laboratory revealed that the USF proteins have antiproliferative activities (11). We showed that USF overexpression specifically inhibited the cellular transformation of primary embryo fibroblasts mediated by c-Myc and activated Ras. Constitutive expression of certain forms of USF also inhibited the proliferation of several transformed cell lines. Our results therefore suggested that one of the normal functions of USF was to protect the cells against the oncogenic potential of Myc (11).

Given these observations, we proposed in this grant to:

Specific aim #1: Investigate the expression of the various USF isoforms in breast cancer cells from different origins.

Specific aim #2: Determine whether overexpression of USF could inhibit the proliferation of breast cancer cells, which would open a novel opportunity for gene therapy.

Completion of these specific aims required the following tasks:

- 1) Obtain different breast cancer cell lines, expand the original aliquot, and freeze for long term conservation.
- 2) Characterize the forms of USF present in different breast cancer cells, using nuclear extracts and electrophoretic mobility shift assays.
- 3) Optimize transfection methods to be used with different breast cancer cell lines.
- 4) Establish and characterize breast cancer cell lines overexpressing USF.

As detailed below, we have accomplished all of these tasks. Furthermore, we demonstrated the existence of a frequent loss of USF function in breast cancer cells as compared to normal breast epithelial cells. This result has opened entirely new areas of investigations and can eventually lead to the discovery of a new tumor suppressor gene.

RESULTS

A. Tasks accomplished

Task 1: We have cultured, frozen aliquots, and analyzed eight normal and breast cancer cell lines (MCF-10A, HBL100, MCF-7, BT-20, Hs578T, T47D, MDA-MB 231 and MDA-MB-468).

Task 2: We have prepared nuclear extracts from these different cell lines and analyzed the levels of endogenous USF using Western blot analysis and electrophoretic mobility shift assay (EMSA).

Task 3: We have investigated the efficiency of different transfection methods and succeeded in optimizing conditions for seven of these cell lines.

Task 4: Using these optimized conditions, we have carried out both transient and stable transfection assays to analyze the effect of USF overexpression in these different cell lines.

B. Expression of the different USF isoforms in various breast and breast cancer cell lines (specific aim 1)

Two different genes, *Usf1* and *Usf2*, encode all ubiquitously expressed USF proteins (10, 11). Analysis by Western blotting and EMSA did not reveal any significant difference in the absolute or relative abundance of the USF1 and USF2-containing homo- and heterodimers in various normal and breast cancer cell lines. The expression of USF

in breast epithelial cells was furthermore very similar to that previously characterized in other human cell lines such as HeLa and Saos-2 (Fig. 1). Eight different breast cell lines, originating from either tumors or normal tissue, were included in this analysis. From these results, we concluded that, if the USF transcription factors were implicated in breast carcinogenesis, changes in the expression or DNA-binding activity of USF were probably not involved.

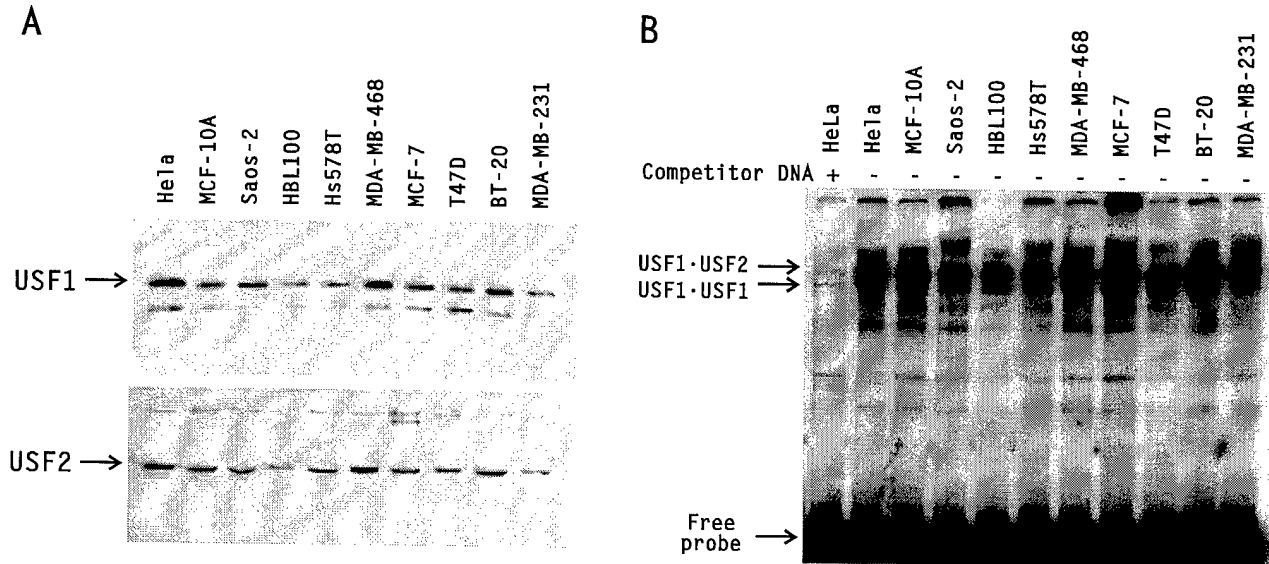


Figure 1: Expression and DNA-binding activity of the USF proteins in eight different breast cell lines. Mini nuclear extracts (13) were prepared for each cell line under identical conditions. HeLa and Saos-2 cells, where the activity of USF is currently best characterized, were included for direct comparison. Extracts were made in each case from 10^6 cells and identical volumes of nuclear extracts were used in each lane.

(A) Western blot analysis. USF1 and USF2 levels were analyzed by probing the same blot successively with USF2- and USF1-specific rabbit peptide antibodies (Santa Cruz Biotechnology). Migration of the 43-kDa USF1 and 44-kDa USF2 polypeptides is indicated at left.

(B) Analysis of endogenous USF DNA-binding activity by EMSA. DNA-binding reactions were assembled using a 150-bp radiolabeled DNA fragment containing a USF-specific binding site. This probe and electrophoresis conditions were chosen to optimize resolution of the different USF dimers (12). Competitor DNA under the form of a 30-bp oligonucleotide containing the USF consensus binding site was included in the reactions as indicated. Migration of the major USF complexes present in all cell lines is indicated at left.

C. Effect of USF overexpression in breast cancer cell lines (specific aim 2)

1) Optimized conditions for transfecting different breast cell lines: We have explored the use of different transfecting agents at various concentrations and with different incubation times in order to optimize our transfection efficiencies in each cell line. Conditions yielding high transfection levels are summarized in Table 1. Transfection efficiencies in BT-20 cells were extremely low under all conditions tested, so this cell line was not used to investigate the effects of exogenous USF expression.

Cell line	Number of cells per well	Transfecting agent	Total DNA	Incubation time
MCF-10A	7×10^5	Lipofectamine (10 μ l)	2 μ g	5 h
Hs578T	2.5×10^5	Lipofectamine (7.5 μ l)	2 μ g	5 h
HBL100	5×10^5	Fugene 6 (3 μ l)	2 μ g	46-50 h
MDA-MB-468	7×10^5	Fugene 6 (6 μ l)	2 μ g	46-50 h
MCF-7	2.5×10^5	Lipofectin (5 μ l)	2.5 μ g	6 h
MDA-MB-231	5×10^5	Fugene 6 (6 μ l)	1 μ g	46-50 h
T47D	7×10^5	Fugene 6 (3 μ l)	2 μ g	46-50 h

Table 1: Conditions yielding efficient transfections in various breast cell lines. Prior to transfection, the indicated number of cells were seeded in 6-well plates. The transfecting agent and total amount of transfected plasmid DNA, as well as the length of the incubation period during which the cells were left in contact with the DNA and transfecting agent, are also indicated. For transient transfection assays, the cells were collected 46-50 hours after transfection and extracts prepared for luciferase assay.

Using these optimized conditions, we compared the transfection efficiency of the different cell lines using the pRSV-Luc reporter plasmid in which luciferase expression is driven by the ubiquitously active Rous sarcoma virus promoter. These experiments revealed that the transfection efficiencies were not too dissimilar, with less than 4 fold difference between the largest and smallest values of luciferase activity (Fig. 2). HBL100 and MDA-MB-468 were the two cell lines that transfected with the lowest efficiency.

EMSA was used to monitor our ability to overexpress USF in the same cell lines. In all cases, high overexpression was observed after transient transfection of expression vectors encoding either USF1, USF2, or the deletion mutant USF2 Δ N (Fig. 3). The latter

construct contains only the DNA-binding domain of USF2 and was useful to determine the expression of reporter genes in the absence of USF transcriptional activity.

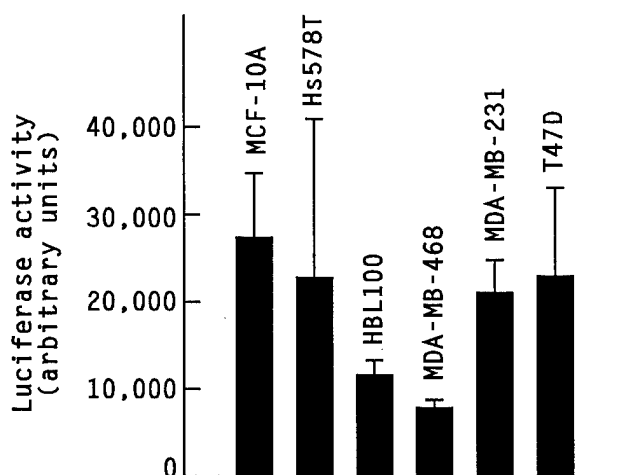


Figure 2: Activity of the RSV-Luc reporter in different breast cell lines. Transfections were carried out as described in Table I. The resulting luciferase activities were monitored using a scintillation counter and are expressed in arbitrary units.

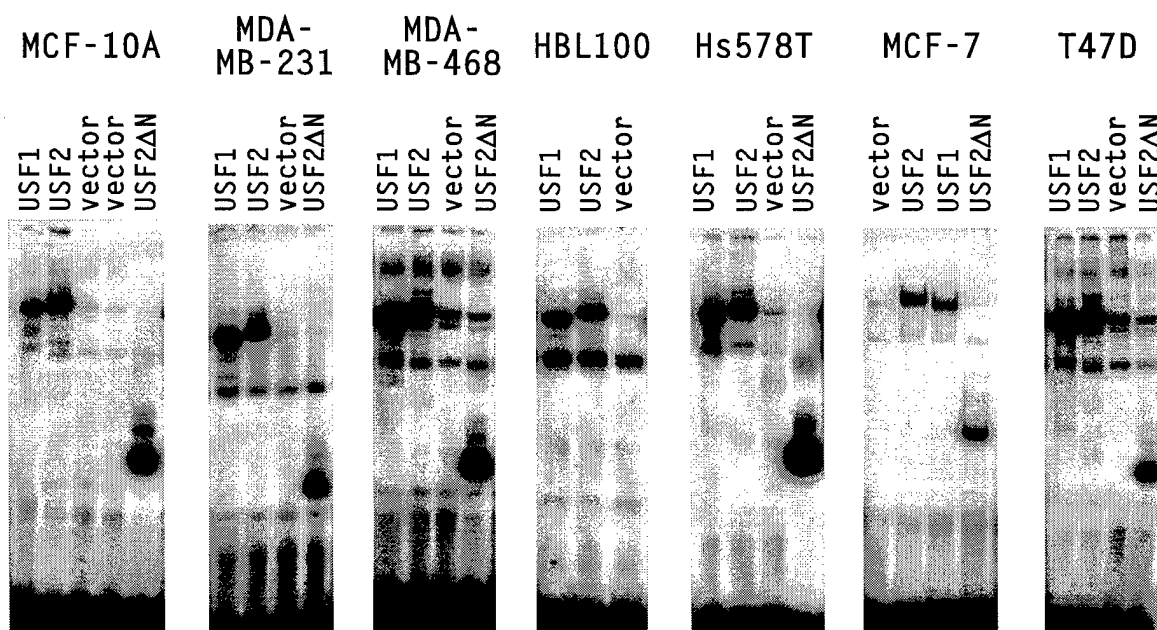


Figure 3: Expression of exogenous USF in different breast cell lines. Cells were transfected as described in Table I with expression vectors encoding either USF1, USF2, or USF2ΔN, or with the corresponding empty vector, as indicated above each lane. USF overexpression was monitored by EMSA using a radiolabeled 33-bp oligonucleotide containing the USF consensus binding site.

2) Transcriptional activity of USF in normal and breast cancer cell lines: To investigate the transcriptional activity of USF in the different cell lines, we used transient cotransfection assays with the reporter plasmids shown in Fig. 4A. Both pMLLuc and pU3MLLuc contain the adenovirus major late minimum promoter driving transcription of the luciferase gene. The two reporters differ only in the presence or absence of 3 USF-specific binding sites inserted in pU3MLLuc upstream of the TATA box. Our earlier studies had shown that the activity of the pU3MLLuc reporter is strongly activated in HeLa cells by cotransfection with either USF1 or USF2 (14).

a. Transcriptional activity of USF in normal breast epithelial cells: We used MCF-10A, a spontaneously immortalized cell line, to evaluate the transcriptional activity of USF in normal breast epithelial cells (Fig. 4). In these cells, transfection of the pU3MLLuc reporter yielded a luciferase activity 13 fold higher than that observed with pMLLuc, indicating that endogenous proteins activated transcription by binding to the USF sites (Fig. 4B). To determine whether these endogenous proteins were USF itself, we cotransfected expression vectors encoding dominant negative mutants for either the USF or the Myc transcription factors. A-USF and A-Max are constructs in which the basic region of USF1 or Max, respectively, was replaced by an acidic sequence (15, 16). This substitution greatly stabilizes heterodimer formation. For example, at a 3:1 ratio, A-USF essentially abolishes DNA binding by USF in vitro (15). Cotransfection of A-USF significantly reduced the activity of the pU3MLLuc reporter in MCF-10A cells, while cotransfection of A-Max was much less inhibitory. Cotransfection of either A-USF or A-Max had essentially no effect on the activity of the pMLLuc reporter (Fig. 4B). From these results, we concluded that endogenous USF, rather than Myc-related proteins, was primarily responsible for the activity of the pU3MLLuc reporter in MCF-10A cells.

The ability of exogenous USF proteins to function as transcriptional activators in MCF-10A cells was monitored by cotransfecting pU3MLLuc and expression vectors for either USF1, USF2, or the USF2 Δ N mutant lacking all transcriptional activation domains. As shown in Fig. 4C, transcription from the pU3MLLuc reporter was increased by cotransfection of USF1 and very strongly increased by cotransfection of USF2, indicating that exogenous USF proteins were also active in MCF-10A cells. In contrast, cotransfection of USF2 Δ N strongly repressed the transcription of pU3MLLuc, probably by preventing the binding of endogenous USF to the promoter DNA (Fig. 4C). From these experiments, we concluded that both endogenous and exogenous USF proteins were active transcription factors in normal breast epithelial cells.

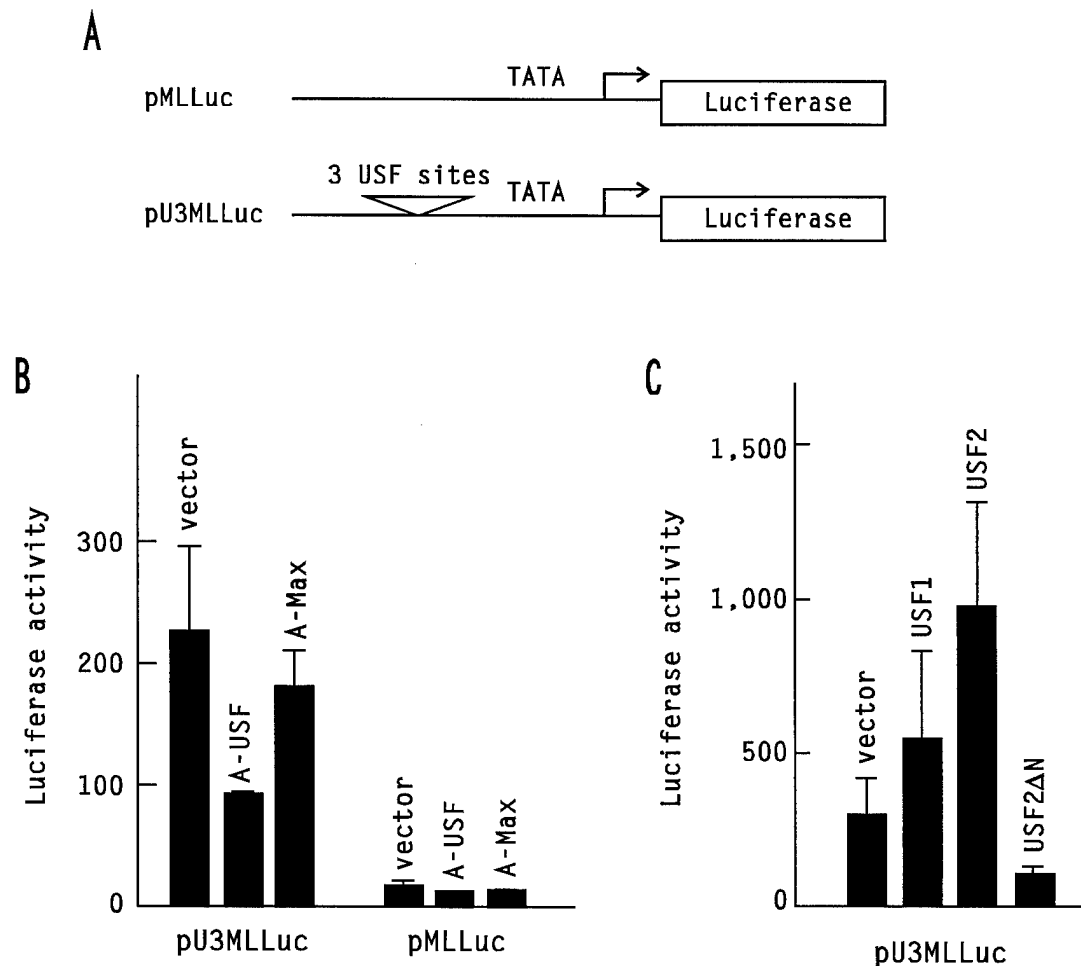


Figure 4: The USF proteins are transcriptionally active in normal breast epithelial cells.

(A) Schematic representation of the reporter plasmids. (B) Transcriptional activity of endogenous USF in MCF-10A cells was monitored by transfecting either the pMLLuc or the pU3MLLuc reporter in the presence or absence of dominant negative mutants of the USF or Myc transcription factors, as indicated. (C) The transcriptional activity of exogenous USF1, USF2 or USF2ΔN in MCF-10A cells was monitored by cotransfection with the pU3MLLuc reporter.

b) Evidence for a partial or complete loss of USF transcriptional activity in breast cancer cell lines: In contrast to the pRSV-Luc reporter, which yielded comparable luciferase activities in all cell lines (Fig. 2), the USF-dependent pU3MLLuc reporter was very poorly transcribed in all six tumorigenic breast cell lines tested as compared to MCF-10A cells (Fig. 5). This was surprising since USF was present at similar levels in all these cell lines (Fig. 1). Given the evidence for a strong transcriptional activity of endogenous USF in MCF-10A (Fig. 4B), these results suggested that endogenous USF exhibited a decreased transcriptional activity in cancer cells as compared to normal cells.

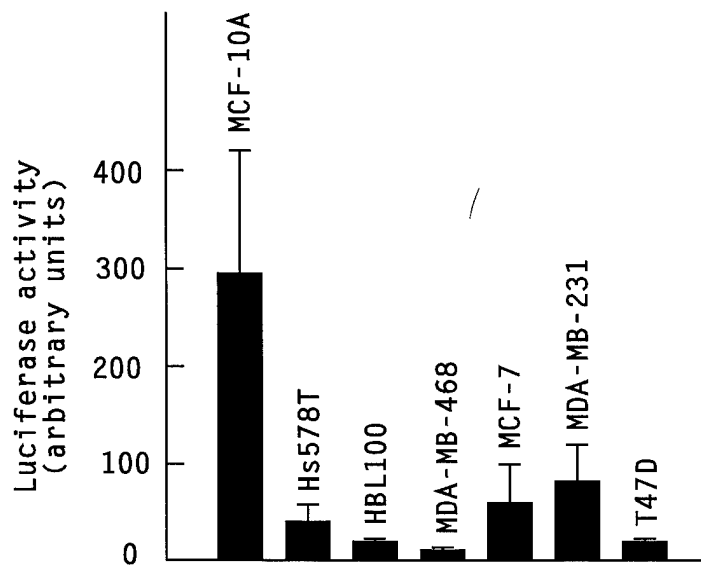


Figure 5: Activity of endogenous transcription factors at the pU3MLLuc reporter in different cell lines.

The ability of exogenous USF1 and USF2 to activate transcription in different breast cancer cell lines was next analyzed by cotransfection assay using the pU3MLLuc reporter and USF2 Δ N as a negative control (Fig. 6). In three cell lines, Hs578T, MDA-MB-468 and T47D, strong activity was detected for USF1 (13 to 22 fold activation), while USF2 stimulated transcription only 2 to 3 fold over the endogenous level. Given that USF2 was actually more active than USF1 in MCF-10A, this result suggested a deficiency affecting specifically the transcriptional activity of USF2 in these 3 cell lines. Interestingly, USF1 and USF2 were both inactive in MCF-7, MDA-MB-231 and HBL100. This result indicated a loss of USF function in these cell lines very similar to that previously characterized in our laboratory for the Saos-2 osteosarcoma cell line (15). In Saos-2 cells, the inactivity of the USF proteins was linked to the inactivity of the USF-specific region (USR), a transcriptional activation domain that is highly conserved in USF1 and USF2 (15). Together, these observations can be explained if a specialized coactivator, which normally interacts with the USR domain to mediate the transcriptional activity of both USF1 and USF2, is absent in Saos-2 cells as well as in several breast cancer cell lines. If this explanation is correct, the loss of this USF-specific transcriptional coactivator may well play a key role in carcinogenesis. Therefore, cloning and identification of the corresponding gene has now become a major goal in our laboratory.

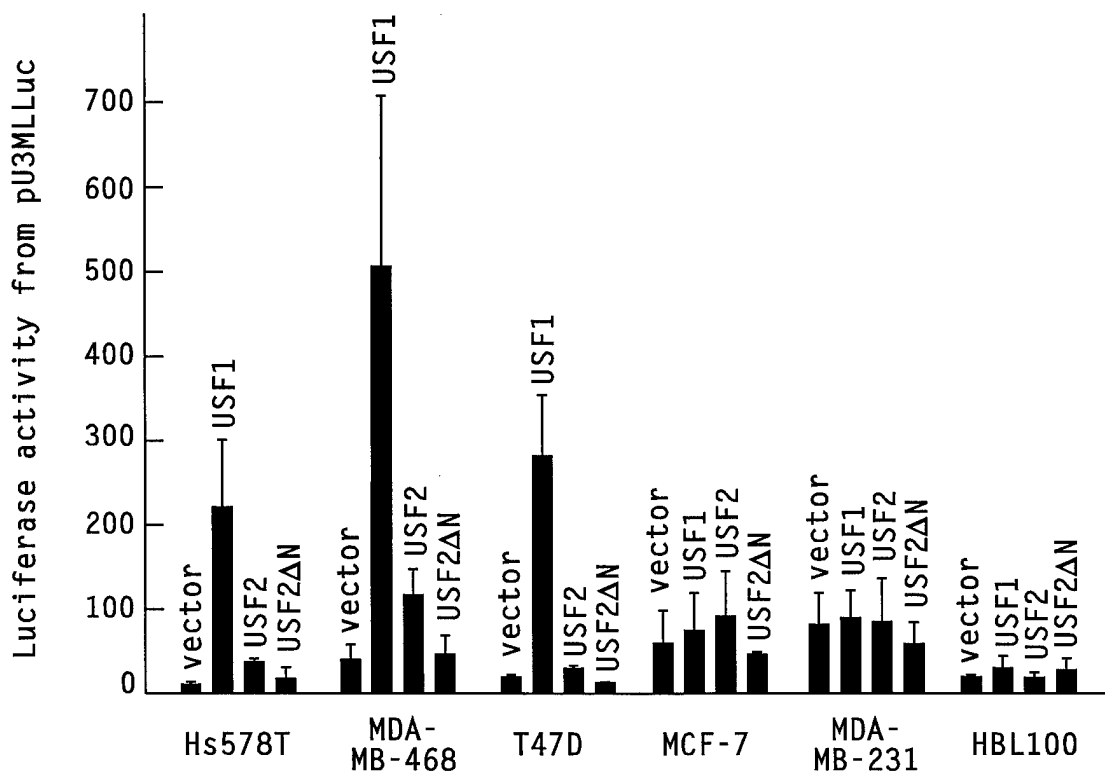


Figure 6: Transcriptional activity of exogenous USF in the different cell lines. The transcriptional activity of exogenous USF proteins in different cell lines was determined by cotransfecting pU3MLLuc with the indicated USF expression vectors.

c) Effect of USF overexpression on the proliferation of normal and breast cancer cell lines: In HeLa cells, where the USF proteins are transcriptionally active, overexpression of USF2 was found to drastically inhibit growth (11, 15). Therefore, when we initiated the studies reported here, one of our goals was to determine whether overexpression of USF proteins, and in particular overexpression of USF2, could similarly inhibit the proliferation of breast cancer cells. However, in HeLa cells, growth inhibition by USF2 requires it to be transcriptionally active (11). Given the general loss of USF2 transcriptional activity in breast cancer cells (Fig. 6), it seemed therefore unlikely that USF2 overexpression would be growth inhibitory in these cells. Nevertheless, we carried out a few experiments using colony formation assays to monitor the effect of USF overexpression on proliferation. Overexpression of USF1 or USF2 had essentially no effect on the colony plating ability of MCF-10A, Hs578T, or MCF-7 (Table 2). In Hs578T and MCF-7 cells, the lack of effect of USF2 on proliferation is probably related to its lack of transcriptional activity, as previously found in the case of the Saos-2 cell

line (15). On the other hand, the ability of MCF-10A cells to proliferate in the presence of elevated levels of transcriptionally active USF2 is interesting since it suggests that the growth inhibition by USF may be specific to transformed cells.

Cotransfected plasmids:	MCF-10A colonies		Hs578T colonies	MCF-7 colonies
	Exp. 1	Exp.2		
pSV2neo	115	179	108	614
pSV2neo + USF1	73	77	187	622
pSV2neo + USF2	80	116	139	583
pSV2neo + USF2ΔN	45	86	183	674

Table 2: Effect of USF overexpression on the proliferation of MCF-10A, Hs578T and MCF-7 cells. Cells were cotransfected with pSV2neo and the indicated USF expression vectors. After 3 weeks of selection in the presence of G418, resistant colonies were stained with crystal violet and counted.

3) Model for the antagonistic roles of USF and Myc in the control of cellular proliferation: Our experiments indicate that a partial or complete loss of USF function is a common feature in cancer cells in general, and in breast cancer cells in particular. Given the antiproliferative properties of the USF proteins in other cell lines and also their ability to antagonize the transforming activity of Myc (11), we like to propose a model in which cellular proliferation is regulated in normal cells by the opposing effects of USF and Myc. In cancer cells, uncontrolled proliferation may be triggered by the overexpression of Myc and/or by the inactivation of USF (Fig. 7). This model accounts for the fact that both Myc overexpression and USF inactivation are common events in cancer cell lines. Whether USF is also inactivated in tumor cells in vivo remains however to be determined.

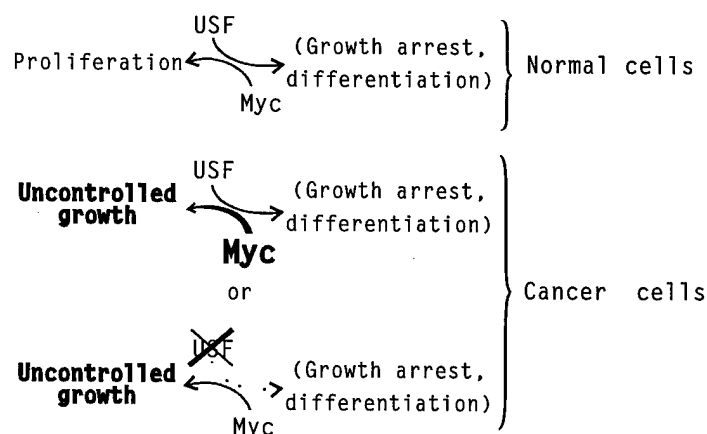


Figure 7: Schematic representation of a model for the antagonism between USF and Myc in the control of cellular proliferation.

CONCLUSIONS

We have analyzed the abundance and activity of the USF transcription factors in normal and tumorigenic breast epithelial cells. Similar USF DNA-binding activities and similar expression levels of the ubiquitous USF1 and USF2 polypeptides were observed in all cell lines tested. In MCF-10A, a normal breast epithelial cell line, strong USF transcriptional activity was demonstrated for both endogenous proteins and exogenously expressed USF1 and USF2. In contrast, breast cancer cell lines exhibited either a normal USF1 activity but greatly diminished USF2 activity or a complete loss of transcriptional activity for both USF1 and USF2. This partial or complete loss of USF function in breast cancer cells is likely to contribute to the uncontrolled proliferation and tumorigenicity of these cells. Further studies will be necessary to characterize the genetic alteration(s) that are responsible for the inactivity of USF in different breast cancer cells and determine whether these alterations involve a novel tumor suppressor gene.

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PUBLICATIONS

Articles:

Financial support from the Department of the Army will be acknowledged in at least two publications:

- 1) Qyang, Y., T. Lu, P.M. Ismail, D. Krylov, C. Vinson and M. Sawadogo. Cell type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation (a revised version of this manuscript is currently under review for publication in Mol. Cell. Biol.).
- 2) Ismail, P.M., T. Lu, and M. Sawadogo. Partial or complete loss of USF transcriptional activity in breast cancer cell lines. (manuscript currently in preparation).

Abstracts:

- 1) Sawadogo, M., Ismail, P. and Lu, T. Activity of USF in breast cancer cell lines. Department of Defense Breast Cancer Research Program Meeting Era of Hope, Washington, DC, November 1997.

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- 3) Yibing Qyang, M.S. (graduate student, 5% effort on this project)
- 4) Hui-Xin Yang, M.S. (Research Assistant I, 5% effort on this project)